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Original Article

Dual role of polyamines in heart ischemia/reperfusion injury through regulation of mitochondrial permeability transition pore

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Abstract: Polyamines (putrescine, spermidine, and spermine) are essential polycations that play important roles in various physiological and pathophysiological processes in mammalian cells. The study was to investigate their role in cardioprotection against ischemia/reperfusion (I/R) injury and the underlying mechanism. Isolated hearts from male Sprague-Dawley rats were Langendorff-perfused and cardiac I/R was achieved by 30 min of global ischemia followed by 120 min of reperfusion. Different concentrations of polyamines (0.1, 1, 10, and 15 μmol/L of putrescine, spermidine, and spermine), cyclosporin A (0.2 μmol/L), or atractyloside (20 μmol/L) were given 10 min before the onset of reperfusion. The hemodynamics were monitored; the lactate dehydrogenase (LDH) levels in the coronary effluent were measured spectrophotometrically; infarct size was determined by the 2,3,5-triphenyltetrazolium chloride staining method; and mitochondrial permeability transition pore (MPTP) opening was determined spectrophotometrically by the Ca^{2+}-induced swelling of isolated cardiac mitochondria. The results showed that compared to I/R alone, 0.1 and 1 μmol/L polyamines treatment improved heart function, reduced LDH release, decreased infarct size, and these effects were inhibited by atractyloside (MPTP activator). In isolated mitochondria from normal rats, 0.1 and 1 μmol/L polyamines treatment inhibited MPTP opening. However, 10 and 15 μmol/L polyamines treatment had the opposite effects, and these effects were inhibited by cyclosporin A (MPTP inhibitor). Our findings showed that polyamines may have either protective or damaging effects on hearts suffering from I/R by inhibiting or activating MPTP opening.

Key words: heart; ischemia/reperfusion; mitochondrial permeability transition pore; polyamines

多胺通过调节线粒体通透性转换孔在心肌缺血再灌注损伤中发挥双重作用

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摘 要：多胺(腐胺、亚精胺和精胺)是一类重要的聚阳离子化合物，在哺乳动物各种生理和病理过程中起重要作用。本研究旨在探索多胺(腐胺、亚精胺和精胺)在心肌缺血再灌注(ischemia/reperfusion, I/R)损伤中的作用及其机制。采用Langendorff离体心脏灌流装置对大鼠离体心脏进行灌流，全心缺血30 min，再灌注120 min。在复灌前10 min给予不同浓度的多胺(0.1、1、10、15 μmol/L腐胺、亚精胺和精胺)、环孢菌素A (0.2 μmol/L)或苍术苷(20 μmol/L)。记录血流动力学变化；分光光度法检测灌流液中乳酸脱氢酶(lactate dehydrogenase, LDH)含量；TTC染色法测定心肌梗死面积；分离心肌线粒体，Ca^{2+}诱导肿胀，分光光度计测定线粒体通透性转换孔(mitochondrial permeability transition pore, MPTP)的开放程度。结果显示，与单独的I/R相比，0.1和1 μmol/L多胺处理改善大鼠心脏功能，降低LDH释放，减少心肌梗死面积，但这些作用被MPTP开放剂苍术苷抑制。在分离自正常大鼠的线粒体，0.1和1 μmol/L多胺处理抑制了MPTP的开放。10和15 μmol/L多胺处理却出现相反的作用，

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这些作用被MPTP抑制剂环孢菌素A抑制。以上结果表明，多胺既可通过抑制MPTP减轻心肌I/R损伤，又可通过促进MPTP开放加重心肌I/R损伤。

关键词：心脏；缺血再灌注；线粒体通透性转换孔；多胺
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Polyamines (putrescine, spermidine, and spermine) are essential polycations that are synthesized by nearly all living cells. They play important roles in various physiological and pathophysiological processes in mammalian cells, such as proliferation, differentiation, intracellular signaling, and apoptosis [1, 2]. Accumulating evidence has shown that they play a critical role in ischemia/reperfusion (I/R) injury of some tissues, such as kidney, brain, and heart [3–5]. In cardiac I/R injury, the activities of both ornithine decarboxylase (ODC) and spermidine/spermine N1-acetyltransferase (SSAT), the critical metabolic enzymes of polyamines, are affected. ODC increases during the ischemic period but decreases after reperfusion, and SSAT is up-regulated both during and after reperfusion; these are accompanied by an accumulation of putrescine and decreases in spermidine and spermine concentrations [5]. Although the polyamine system is indeed involved in I/R injury, its exact role is still unclear. Our previous work showed that exogenous spermine increases the injury during heart I/R [6], and a report has shown that polyamines induce cytochrome c release from mitochondria to induce apoptosis in cardiac myocytes [7]. However, other reports have shown that exogenous spermine decreases the injury caused by cardiac I/R [5, 8]. These controversial results indicate that the exact role of spermine in the cardiac I/R process needs further examination. In addition, although decreased production of the other two polyamines (spermine and spermidine) by inhibition of the critical rate-limiting enzyme of polyamine metabolism suggests their involvement in cardiac I/R injury [5], there is still a lack of direct evidence for the exact role of these polyamines in cardiac I/R injury.

The mitochondrial permeability transition pore (MPTP) is a non-specific pore located in the inner membrane of the mitochondrion that plays an important role in cardiac I/R injury [9]. The MPTP regulates cellular Ca^{2+} homeostasis, cell death, and apoptosis, as well as the start or termination of I/R injury [10]. Inhibition of MPTP opening during reperfusion has a cardioprotective effect, while opening of the MPTP during reperfusion increases the injury caused by I/R [11–14]. To date, the endogenous factors involved in the regulation of MPTP opening during I/R remain unknown.

Based on the above, we postulated that polyamines may be involved in cardiac I/R injury via the regulation of MPTP opening. To test this hypothesis, we designed experiments to determine the role of the polyamines (putrescine, spermidine, and spermine) in isolated Langendorff-perfused rat hearts suffering from I/R, and investigated their possible association with the MPTP using isolated cardiac mitochondria.

1 MATERIALS AND METHODS

1.1 *Animals*

Male Sprague-Dawley rats (240–290 g) were obtained from the Animal Center of Tongji Academy of Medical Sciences. All experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and approved by Ethics Committee for the Use of Experimental Animals in China Three Gorges University .

1.2 *Chemicals*

Putrescine, spermidine, spermine, 2,3,5-triphenyltetrazolium chloride, and other chemicals were from Sigma Chemical Co., St Louis, MO. The lactate dehydrogenase (LDH) kit was from Nanjing Jiancheng Reagent Co., Nanjing, China.

1.3 *Perfusion protocol for Langendorff-perfused rat hearts*

All isolated hearts were allowed to equilibrate for at least 20 min and received 30 min of global ischemia followed by 120 min of reperfusion. A fluid-filled latex balloon was inserted into the left ventricle through the left atrium and the balloon catheter was linked to a pressure transducer connected to a data acquisition system (Biopac, Goleta, CA) to assess contractile function. The left ventricular end-diastolic pressure (LVEDP) was adjusted to between 6 and 8 mmHg. Left ventricular developed pressure (LVDP) and the maximal rate of rise or fall of left ventricular pressure ($\pm dp/dt_{max}$) were monitored continuously. Polyamines (0.1, 1, 10, and 15 μmol/L of putrescine, spermidine, and sper-

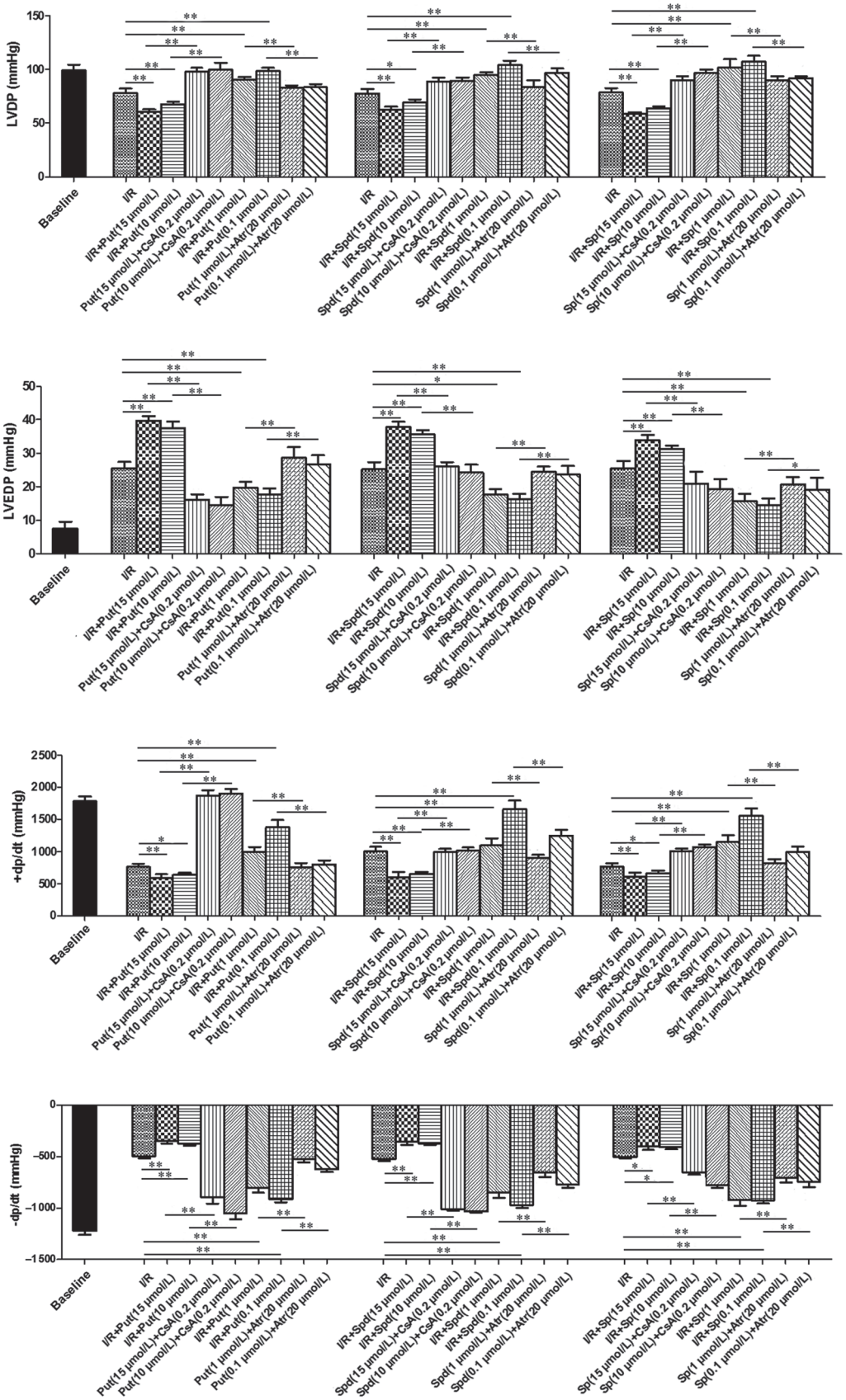

Fig. 1. Recovery of left ventricular function at 30 min of reperfusion in isolated hearts subjected to global ischemia. Put, putrescine; Spd, spermidine; Sp, spermine; LVDP, left ventricular developed pressure; LVEDP, left ventricular end diastolic pressure; ±d*p*/d*t*max, maximal rate of rise or fall of left ventricular pressure. Values are expressed as the mean ± SD; $n = 6$/group. $^{*}P < 0.05$, $^{**}P < 0.01$.

mine) were given 10 min before the onset of reperfusion through the perfusion system. The MPTP inhibitor cyclosporin A (CsA, 0.2 μmol/L) or the MPTP activator atractyloside (Atr, 20 μmol/L) was perfused 10 min before the beginning of reperfusion.

1.4 *Infarct size measurement*

At the end of 120 min reperfusion, each heart was quickly removed and immediately frozen at −20 °C for 3 h. The frozen hearts were cut into 2-mm transverse slices, incubated in 2% w/v triphenyltetrazolium chloride in phosphate buffer, pH 7.4 for 8 min, then fixed in 10% formalin for 10 min. In the risk zone, the viable tissue stained red and the infarcted tissue appeared pale. Infarct and risk zone areas were measured by planimetry with ImageJ (National Institutes of Health, Bethesda, MD). Infarct size was expressed as a percentage of the risk zone.

1.5 *LDH measurement*

The coronary effluent from each isolated Langendorff-perfused heart was collected at 10 min of reperfusion and the LDH activity was spectrophotometrically assayed and expressed as units per liter.

1.6 *Isolation of mitochondria*

Briefly, heart tissue from normal rats was homogenized in ice-cold buffer (160 mmol/L KCl, 10 mmol/L EGTA, pH 7.4, and 0.5% fatty acid-free bovine serum albumin). The homogenate was centrifuged at 1 000 *g* for 10 min at 2 °C, and the supernatant was centrifuged at 8 000 *g* for 10 min at 2 °C. The pellet was re-suspended in buffer containing 320 mmol/L sucrose and 10 mmol/L Tris-HCl (pH 7.4), and centrifuged at 8 000 *g* for 10 min at 2 °C. Then we obtained purified mitochondria, and the mitochondrial protein was quantified with Coomassie brilliant blue [15].

1.7 *Measurement of MPTP opening*

Opening of the MPTP was determined by Ca^{2+}-induced mitochondrial swelling, which was measured as a reduction in the absorbance at 520 nm (A_{520}). Isolated cardiac mitochondria were suspended in swelling buffer (in mmol/L: 120 KCl, 20 MOPS, 5 KH_2PO_4, and 10 Tris-HCl, pH 7.4), and the final concentration was 0.25 mg/mL. MPTP opening was induced by adding 200 μmol/L $CaCl_2$ to the mitochondria, and the A_{520} was measured spectrophotometrically [16, 17]. The effect of the polyamines on MPTP opening was assessed by adding different concentrations of polyamines to the mitochondrial solution before addition of $CaCl_2$. The absorbance changes were measured every 10 s for a total of 15 min.

1.8 *Statistical analysis*

All values are expressed as the mean ± SD. Statistical comparisons were performed by one-way analysis of variance and the Newman-Keuls test for post-comparison of groups. The significance level was set at $P < 0.05$.

2 RESULTS

2.1 Hemodynamics

In the I/R group, LVDP and ±d*p*/d*t*max were decreased at 30 min of reperfusion, while LVEDP was elevated. Polyamines at 10 or 15 μmol/L decreased LVDP, ±d*p*/d*t*max, and increased LVEDP, which had more serious injury than I/R alone, and these effects were abolished by CsA (0.2 μmol/L), an MPTP inhibitor. Polyamines at 0.1 or 1 μmol/L resulted in increased LVDP and ±d*p*/d*t*max and a relatively decreased LVEDP compared to I/R alone, and this effect was abolished by Atr (20 μmol/L), an MPTP opener (Fig. 1).

2.2 LDH and myocardial infarct size

Relatively high concentrations (10 and 15 μmol/L) of polyamine treatment had more LDH release and enlarged infarct size than I/R alone group, and these effects were greatly reduced by treatment with CsA (0.2 μmol/L), a specific MPTP inhibitor (Fig. 2 and Fig. 3). The infarct size and LDH content in the coronary effluent were greatly reduced by treatment with relatively low concentrations of polyamines (0.1 and 1 μmol/L) compared to the I/R group, and these effects were abolished by treatment with Atr (20 μmol/L), a specific MPTP inhibitor (Fig. 2 and Fig. 3).

2.3 Effects of polyamines on the MPTP

In mitochondria isolated from normal rat hearts, polyamines at 0.1 and 1 μmol/L significantly inhibited Ca^{2+}-induced mitochondrial swelling, an index of MPTP opening. On the contrary, polyamines at 10 and 15 μmol/L decreased it, i.e. reduced MPTP opening (Fig. 4).

3 DISCUSSION

The results from the present study indicated that polyamines can either decrease or increase injury of the heart by I/R, and the regulation of MPTP opening by polyamines may play an important role in this process.

Changes in polyamine content during I/R have been reported by Zhao *et al*. [5]. Our previous study also

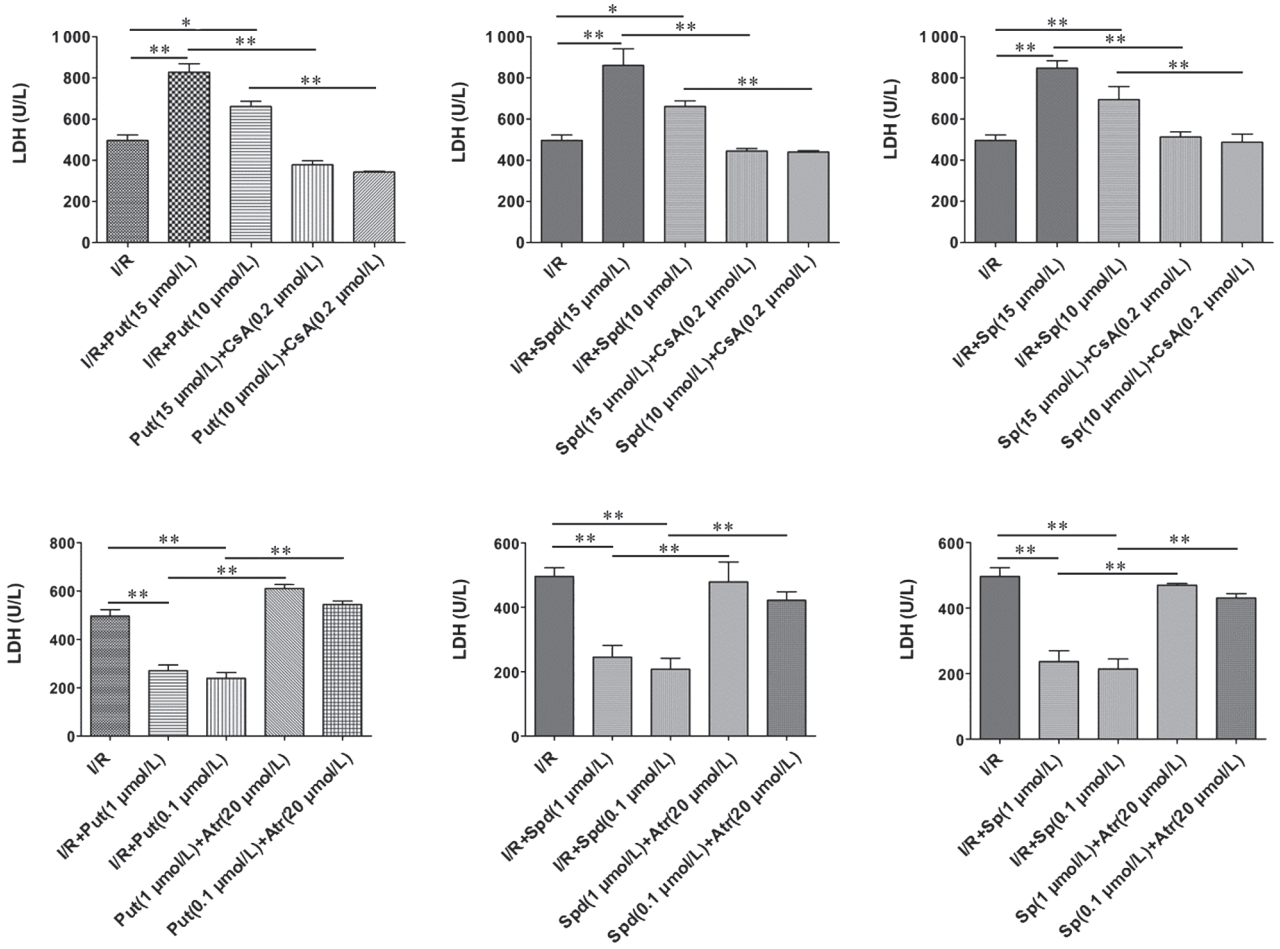

Fig. 2. Effects of polyamines on LDH release at 10 min of reperfusion in isolated rat hearts subjected to I/R. Put, putrescine; Spd, spermidine; Sp, spermine. Mean ± SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$.

showed that putrescine, spermidine, and spermine decrease in the heart during I/R, suggesting that supplementation of polyamines during I/R may have protective effects on the heart. In addition, several research groups have reported that pretreatment with spermine protects the heart and brain against I/R injury [4, 5]. However, our previous work and other reports have shown that exogenous spermine increases the I/R injury of the heart [6, 7]. It was difficult to find a good explanation for these conflicting results. Therefore, in order to further determine the exact role of polyamines in I/R injury of the heart, in the present study we administered the polyamines at different concentrations prior to reperfusion through the Langendorff perfusion system. The results showed that the relatively low concentrations of polyamines (0.1 and 1 μmol/L) decreased the injury caused by I/R with improved hemodynamics along with decreased myocardial infarct size and LDH release. However, the relatively high concentrations (10 and 15 μmol/L) increased the injury caused by I/R. Thus, compared to previous reports with cultured cardiac myocytes, which showed polyamines given exogenously can be cardioprotective in I/R [18], our results showed dual effects of polyamines on hearts suffering from I/R in a dose-dependent manner.

The MPTP is a non-selective pore located in the inner membrane of the mitochondria. It has been demonstrated by many studies that the MPTP plays an important role in cardiac I/R injury. Opening of the MPTP during reperfusion leads to apoptosis of cardiac cells, and inhibition of MPTP opening reduces the injury caused by I/R [11]. Our previous studies have also shown the same results [19–21]. Based on the results reported here, we proposed that polyamines play their dual role in heart I/R injury via the regulation of MPTP opening. In order to test this hypothesis, we further investigated the effects of polyamines on MPTP opening in mitochondria isolated from normal rat hearts. The results showed

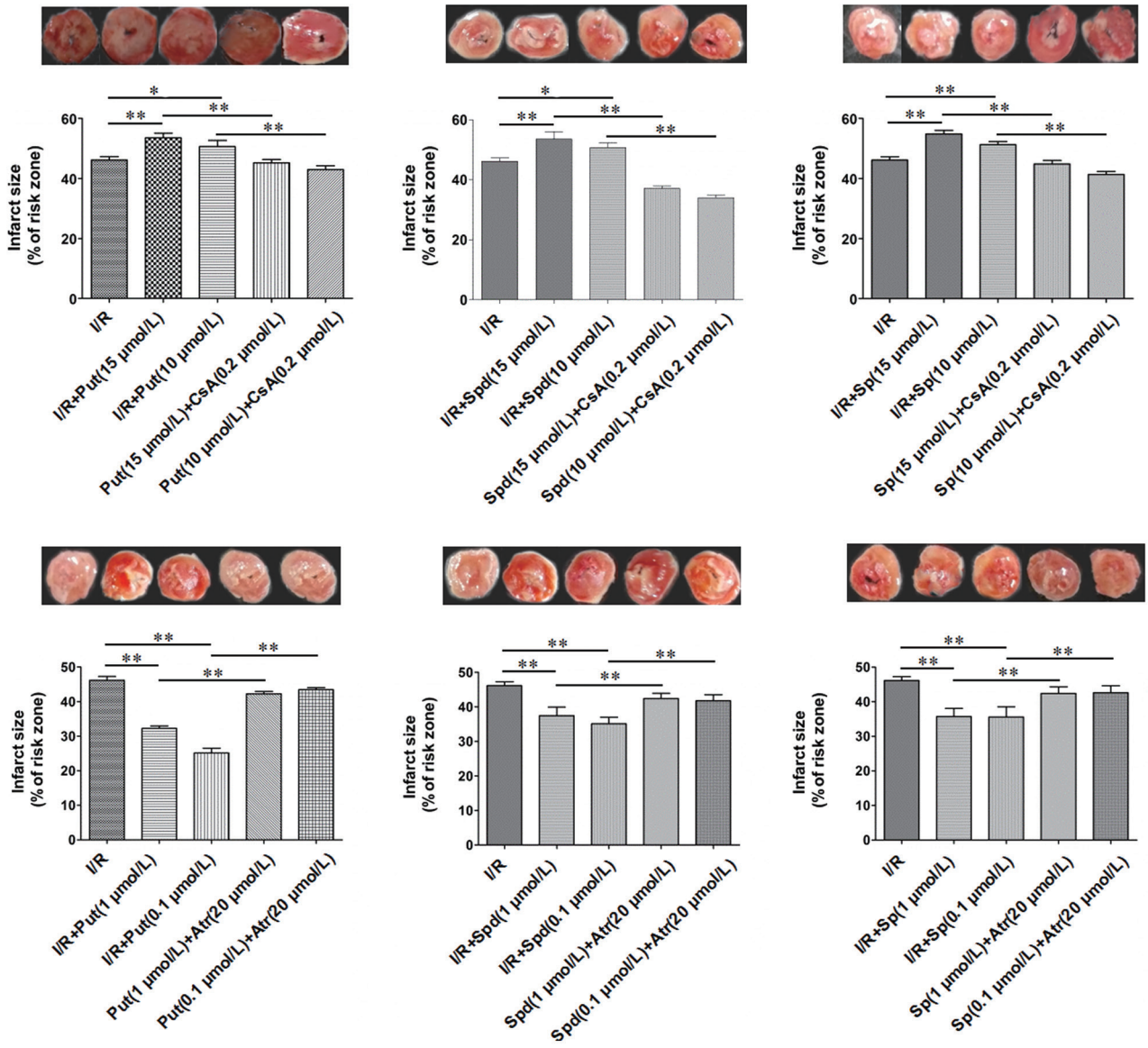

Fig. 3. Effects of polyamines on infarct size in isolated rat hearts subjected to I/R. Put, putrescine; Spd, spermidine; Sp, spermine. Mean ± SD, $n = 6$. $^{*}P < 0.05$, $^{**}P < 0.01$.

that polyamines at relatively low concentrations (0.1 and 1 μmol/L) inhibited the MPTP opening induced by Ca^{2+}. However, polyamines at relatively high concentrations (10 and 15 μmol/L) increased the MPTP opening induced by Ca^{2+}, suggesting that the cardioprotection provided by low concentrations of polyamines after I/R may occur via inhibiting MPTP opening, while relatively high concentrations may increase the I/R injury via increasing MPTP opening.

In order to further determine whether the effects of polyamines on heart I/R injury are via the MPTP, we did additional experiments to test whether the dual effects of polyamines can be abolished by Atr, an MPTP opener, or CsA, an inhibitor of MPTP opening. The results showed that the cardioprotection provided by low concentrations of polyamines was abolished by Atr, and the effects of high concentrations of polyamines on I/R of hearts were abolished by CsA. Therefore, polyamines can have either protective or injurious effects via inhibiting or activating MPTP opening during cardiac I/R. Reports have shown that opening of MPTP results in a decreased MnSOD level along with increased ROS activity and myocardial cell apoptosis, and that inhibition of MPTP reverses these effects [22–24], so low concentrations of polyamines may provide cardioprotection by inhibiting oxidative stress, while high

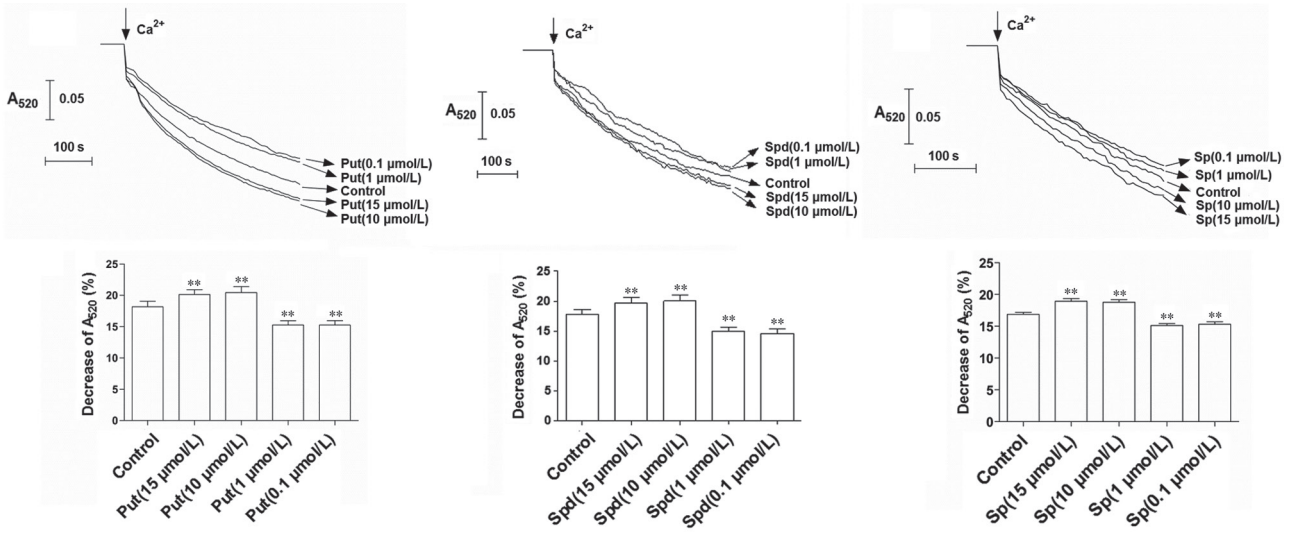

Fig. 4. Effects of different concentrations of polyamines on MPTP opening. MPTP opening was measured by spectrophotometric monitoring of the decrease in absorbance at 520 nm (A_{520}) after addition of $CaCl_2$ (200 μmol/L). Put, putrescine; Spd, spermidine; Sp, spermine. Mean ± SD, $n = 6$. $^{**}P < 0.01$ *vs* Control.

concentrations of polyamines may increase I/R injury by increasing oxidative stress.

The regulatory effects of polyamines on the MPTP were examined in mitochondria isolated from cardiac myocytes, which excluded other factors that may contribute to regulation of the MPTP. This result suggested that MPTP opening by polyamines is a direct rather than an indirect action. However, the exact regulatory site of polyamines on the MPTP needs further investigation. Cyclophilin D is one of the main constituents of the MPTP, and plays an important role in its regulation. Whether cyclophilin D is the point of direct regulation by polyamines needs further examination.

In summary, these results suggest that polyamines have either protective or injurious effects via inhibiting or activating MPTP opening in I/R heart.

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